

# Class A scavenger receptors regulate tolerance against apoptotic cells, and autoantibodies against these receptors are predictive of systemic lupus

Fredrik Wermeling,<sup>1</sup> Yunying Chen,<sup>2</sup> Timo Pikkarainen,<sup>2</sup>  
Annika Scheynius,<sup>1</sup> Ola Winqvist,<sup>1</sup> Shozo Izui,<sup>3</sup> Jeffrey V. Ravetch,<sup>4</sup>  
Karl Tryggvason,<sup>2</sup> and Mikael C.I. Karlsson<sup>1</sup>

<sup>1</sup>Department of Medicine and <sup>2</sup>Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm 171 76, Sweden

<sup>3</sup>Department of Pathology and Immunology, University of Geneva, 1211 Geneva 4, Switzerland

<sup>4</sup>Laboratory of Molecular Genetics and Immunology, The Rockefeller University, New York, NY 10021

**Apoptotic cells are considered to be a major source for autoantigens in autoimmune diseases such as systemic lupus erythematosus (SLE). In agreement with this, defective clearance of apoptotic cells has been shown to increase disease susceptibility. Still, little is known about how apoptotic cell-derived self-antigens activate autoreactive B cells and where this takes place. In this study, we find that apoptotic cells are taken up by specific scavenger receptors expressed on macrophages in the splenic marginal zone and that mice deficient in these receptors have a lower threshold for autoantibody responses. Furthermore, antibodies against scavenger receptors are found before the onset of clinical symptoms in SLE-prone mice, and they are also found in diagnosed SLE patients. Our findings describe a novel mechanism where autoantibodies toward scavenger receptors can alter the response to apoptotic cells, affect tolerance, and thus promote disease progression. Because the autoantibodies can be detected before onset of disease in mice, they could have predictive value as early indicators of SLE.**

## CORRESPONDENCE

Mikael C.I. Karlsson:  
Mikael.Karlsson@ki.se

A specific B cell subtype in the marginal zone of the spleen is thought to be the producer of autoantibodies in several models of autoimmunity (1, 2). These so-called marginal zone B cells (MZBs), essential for defense and early responses against blood-borne pathogens, are phenotypically characterized by high IgM and complement receptor expression (3). As an example of MZB involvement in self-reactivity, B cells are rescued from deletion in the MZB population in mice expressing a B cell receptor with affinity for DNA (4). Autoreactive MZBs can also be activated spontaneously without T cell help, and the role of these B cells as producers of autoantibodies and responders to self-antigens derived from apoptotic cells is supported by several studies (5, 6). A plausible explanation is that the natural MZB repertoire has a low level of self-reactivity that maintains the cells in a preactivated state enabling them to rapidly respond to

pathogens. This feature also makes them sensitive to defective regulation where they may contribute to autoimmunity by being recruited to produce autoantibodies. The MZBs are predominantly noncirculating and reside in the marginal zone in the spleen, an area with a low rate of blood flow enabling efficient trapping of blood-borne antigens. In the marginal zone, the MZBs are in close contact with highly phagocytic macrophages called marginal zone macrophages (MZMs) (2). The MZMs are characterized by high expression of the class A scavenger receptors macrophage receptor with collagenous structure (MARCO) and scavenger receptor A (SR-A) (2). MARCO and SR-A bind a variety of self- and foreign ligands, including modified low-density lipoprotein and bacterial components (2, 7). The expression of these receptors as well as others such as the C-type lectin SIGNR1 and the strategic position of the MZMs make them competent trappers of antigen from the blood and thus an important component in MZB activation.

The online version of this article contains supplemental material.

A source of autoantigens for B cell activation in systemic lupus erythematosus (SLE) is apoptotic cells, and defects in apoptotic cell clearance increase susceptibility to SLE (8, 9). This clearance defect can be detected in the skin, spleen, and circulation, suggesting that putative autoantigen is available at multiple sites. Because activation/selection of autoreactive MZBs needs to include access to autoantigens, we investigated whether apoptotic cells are localized to the marginal zone of the spleen and thereby could be a source for autoantigens in innate B cell activation. We find that i.v. injected apoptotic cells are rapidly trapped by MZMs in the marginal zone of the spleen.

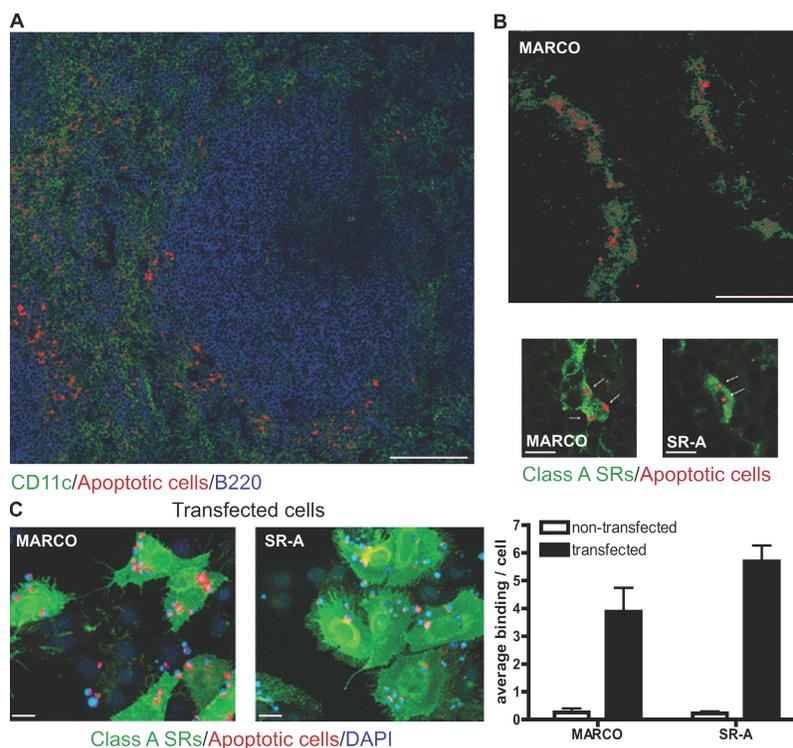
SR-A has been shown to bind apoptotic cells, and we show here that MARCO too has this ability, indicating that these two receptors take part in clearance of apoptotic cells in the marginal zone. When deleting MARCO, SR-A, or both receptors, mice have a lower tolerance threshold and develop higher anti-DNA antibody titres spontaneously and after injection of apoptotic cells. This shows that proper clearance by these receptors in the marginal zone is needed for correct regulation of B cell tolerance. In addition, we find that in  $Fc\gamma RII B^{-/-}$  and (NZB x NZW)F1 mice, which

develop spontaneous SLE, there are autoantibodies produced recognizing both SR-A and MARCO. These antibodies can be detected before onset of disease, suggesting a mechanism where initial break of tolerance against scavenger receptors leads to subsequent alteration in clearance in the marginal zone and finally to disease. The anti-scavenger receptor antibodies are also found in patients with SLE, defining these as a new and potentially clinically relevant marker in human disease.

## RESULTS AND DISCUSSION

### Apoptotic cells are trapped by macrophages in the marginal zone of the spleen

First, we studied if apoptotic cells are localized to the marginal zone of the spleen and could be a source for autoantigens in innate B cell activation. WT mice were injected i.v. with labeled apoptotic cells, and spleens were collected at different time points. We found that the injected apoptotic cells were already trapped by phagocytes in the marginal zone of the spleen 30 min after injection (Fig. 1 A). At 5 h, fewer cells were found, indicating swift clearance in this region (not depicted). Several subtypes of potent APCs reside in the

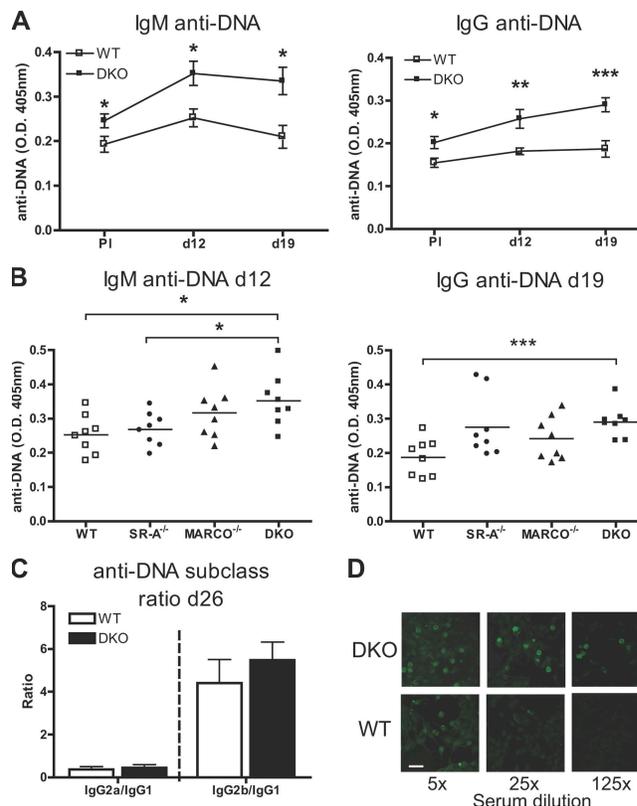


**Figure 1. Apoptotic cells bind MARCO and SR-A, and apoptotic cells are trapped in the marginal zone of the spleen.** (A) PKH26-labeled (red) apoptotic cells were injected i.v. in BALB/c mice, and spleens collected after 30 min were stained with anti-CD11c (DCs; green) and anti-B220 (B cells; pseudo-colored blue). Bar, 100  $\mu$ m. (B; top) A serial cryostat section stained with anti-MARCO (MZMs; green). Bar, 100  $\mu$ m. (Bottom) Higher magnification of MZM-binding apoptotic cells (red) stained with both anti-MARCO and anti-SR-A (green). Arrows indicate apoptotic cells taken up by MZMs. Bar, 10  $\mu$ m. (C) In vitro binding assay using CHO cells transfected with mouse MARCO or SR-A and incubated with labeled apoptotic cells (red). (Left) Cells stained with anti-MARCO or anti-SR-A (green) and DAPI nuclear staining (blue). Bar, 10  $\mu$ m. (Right) Quantification of apoptotic cell binding showing the amount of apoptotic cells binding transfected or nontransfected cells on the same slide. 10–15% of the total amount of CHO cells was successfully transfected. In A and B the Leica confocal system was used, and in C the Leica DMRB microscope was used (refer to Materials and methods).

marginal zone, including DCs, known to be able to ingest apoptotic cells (10, 11). However, even though some apoptotic cells were taken up by CD11c<sup>+</sup> DCs, we found that at early time points, 30 min and 5 h, the apoptotic material primarily bound to MZMs. The cells trapping the apoptotic cells were MZMs, defined in serial sections by their expression of specific scavenger receptors MARCO and SR-A (Fig. 1 B) (2, 12). SR-A has been shown to bind apoptotic cells (13), and when we investigated whether MARCO has this same ability, we found that both receptors could bind apoptotic cells in transfection experiments. In this assay, clustering of apoptotic cells could be seen on MARCO- or SR-A-transfected cells and not on nontransfected cells on the same slide (stained with DAPI) (Fig. 1 C). These results show that MZMs have the ability to trap apoptotic cells in the marginal zone and that both MARCO and SR-A receptors can contribute to the clearance in this region.

### Higher autoantibody responses in scavenger receptor-deficient mice

The finding that MZMs are involved in clearance of apoptotic cells in the marginal zone raised the possibility that they could regulate the response and availability of self-antigens in the marginal zone for recognition by MZBs and DCs. In an analogous system, proper clearance of apoptotic cells by tangible body macrophages has been shown to be important in the germinal center microenvironment where B cells are activated and/or selected (14). Hanayama et al. show that the inability of macrophages to ingest apoptotic cells due to lack of MFG-E8 production in the germinal center leads to autoimmunity. To explore whether MARCO<sup>+</sup> and SR-A<sup>+</sup> macrophages are involved in regulating autoimmune responses, we investigated the ability to maintain tolerance after injection of syngeneic apoptotic cells, without adjuvant, in mice deficient in one or both of these receptors (15, 16). Apoptotic cells were injected weekly four times in WT, SR-A<sup>-/-</sup>, MARCO<sup>-/-</sup>, and double KO (DKO) mice in a protocol adopted from Mevorach et al. (17), and anti-DNA responses were measured with ELISA. All receptor-deficient mice had an elevated and more rapid response to apoptotic cells compared with control mice, and the phenotypes were additive, resulting in the highest response in the DKO mice (Fig. 2 A). Notably, the DKO mice, but not single KO mice, displayed significantly ( $P = 0.01$ ) higher levels of IgM and IgG anti-DNA without provocation by apoptotic cells, suggesting spontaneous development of anti-DNA autoimmune responses (Fig. 2 A and not depicted). However, at the time points tested, we could find no spontaneous development of kidney pathology as measured by proteinuria and histological examination (not depicted). No major differences could be seen with regard to Ig isotype of anti-DNA antibodies in the different KO mice, except that MARCO deficiency tended to contribute more to IgM anti-DNA titres and SR-A deficiency to IgG anti-DNA titres (Fig. 2 B). The specific IgG response was mainly of the IgG<sub>2b</sub> subclass in all mice, and the DKO mice tended to have a higher IgG<sub>2a/b</sub>/IgG<sub>1</sub> ratio than

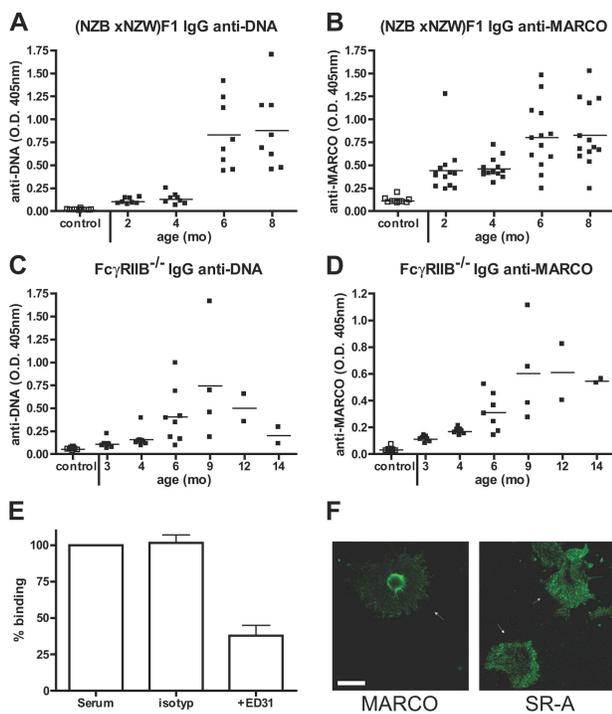


**Figure 2. Class A scavenger receptors regulate tolerance against i.v. injected apoptotic cells.** (A)  $10^7$  syngeneic apoptotic cells were injected i.v. four times weekly in WT and MARCO<sup>-/-</sup>/SR-A<sup>-/-</sup> DKO mice (C57BL/6 background). IgM and IgG anti-DNA responses in serum were measured pre-immune (PI) at days 12 and 19. Data are shown as mean  $\pm$  SEM ( $n = 8$  per genotype). (B) The anti-DNA response in WT, MARCO<sup>-/-</sup>, SR-A<sup>-/-</sup>, and DKO mice at days 12 (IgM) and 19 (IgG). Individual data and mean are presented ( $n = 8$  per genotype). As a control in these experiments, a quantitative analysis of serial dilution of pooled MRL<sup>lpr</sup> sera that develop spontaneous disease revealed that the levels of IgM and IgG anti-DNA antibodies are  $\sim$ 5–40 times that of WT mice injected with apoptotic cells. The PI values for MARCO<sup>-/-</sup> and SR-A<sup>-/-</sup> were not statistically different from WT controls (not depicted). (C) Subclass analysis of the anti-DNA response at day 26, after the fourth injection of apoptotic cells. Data are shown as mean  $\pm$  SEM of the OD 405-nm ratio between IgG<sub>2a</sub>/IgG<sub>2b</sub> and IgG<sub>1</sub> ( $n = 8$  per genotype). (D) Representative ANA pattern from DKO and WT mice after the fourth injection (d26). Bar, 50  $\mu$ m. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (nonparametric Mann-Whitney U-test).

WT, which is suggestive of a higher degree of pathogenicity (Fig. 2 C) (18). In agreement with the anti-DNA ELISA data, antinuclear autoantibody (ANA) titres were also higher in injected DKO mice (Fig. 2 D).

A conceivable explanation for the increased anti-DNA response is that deletion of the receptors leads to a decreased clearance of apoptotic cells, in turn resulting in increased self-antigen load. With this in mind, we investigated whether the mice displayed any defects in the clearance of apoptotic cells. The number of circulating apoptotic cells in the blood did not differ between DKO mice and WT mice, and furthermore

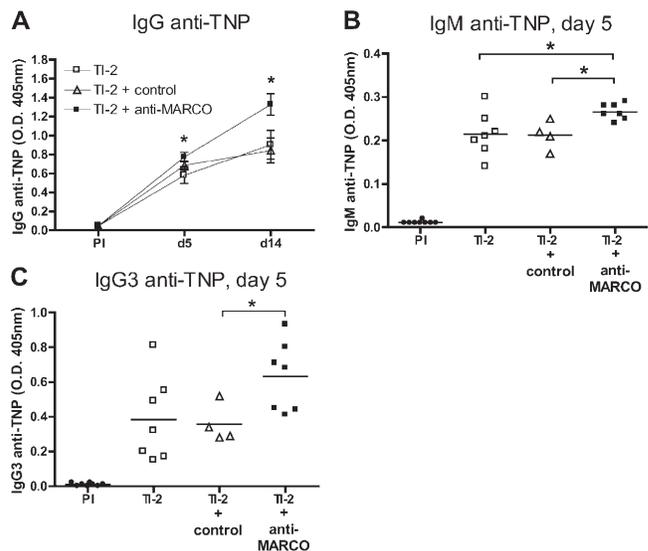
there were no detectable differences in the clearance of i.v. injected apoptotic cells (Fig. S1, available at <http://www.jem.org/cgi/content/full/jem.20070600/DC1>). The lack of difference in systemic clearance suggests a mechanism where specific local change in the response in the marginal zone is responsible for the change in anti-DNA levels. In line with this, the scavenger receptor-deficient mice have a normal MZB population, reduced MZM population, and a higher response to thymus-independent type 2 (TI-2) antigens (unpublished data and reference 16). The fact that deletion of type A scavenger receptors renders mice more susceptible to autoimmunity makes them possible candidates for susceptibility genes in SLE. In mice, the MARCO gene is located on chromosome 1 (122 Mb from the centromere), nearby but slightly proximal to the major lupus susceptibility loci of the NZB, NZW, and BXS mice (19). Nevertheless, one of the BXS loci, *Bxs2*, linked to ANA and anti-DNA production, peaks at the *D1Mit12* marker (122 Mb) in the vicinity of *Marco* (20), suggesting that *Marco* may contribute to SLE susceptibility. The SR-A gene, on chromosome 8 in mice, has not been shown to reside within the known susceptibility loci for SLE or other autoimmune diseases.



**Figure 3. Anti-MARCO antibodies are found in SLE-prone mice.** (A and C) IgG anti-DNA levels in 2–8-mo-old (NZB x NZW)F1 mice and in 3–14-mo-old *FcγRIIB*<sup>-/-</sup> mice. Control mice are 3-mo-old C57BL/6 mice. (B and D) IgG anti-MARCO reactivity in 2–8-mo-old (NZB x NZW)F1 mice, 3–14-mo-old *FcγRIIB*<sup>-/-</sup> mice, and 3-mo-old C57BL/6 mice. (E) Binding to MARCO in the anti-MARCO ELISA blocked with a rat monoclonal antibody (ED31) against MARCO, but not with an isotypic control ( $n = 2$ ). (F) CHO cells transfected with mouse MARCO or SR-A stained with sera from (NZB x NZW)F1 mice and anti-mouse IgG-FITC. Arrows indicate transfected cells stained by the mouse sera. Bar, 20  $\mu$ m.

### Class A scavenger receptors are autoantigens in mouse and man

The ability to bind apoptotic cells and participate in clearance is a characteristic that SR-A and MARCO have in common with the structurally related complement protein C1q (21). In SLE, autoantibodies toward C1q can be found, and data suggest that they increase the severity of glomerulonephritis, but there is also the possibility that they may affect clearance of apoptotic cells (22). With these disease processes in mind, the question was raised if scavenger receptors in a similar manner could function as autoantigens and blocking antibodies could interfere with their clearance function. To investigate possible development of autoantibodies binding class A scavenger receptors, sera from lupus-prone (NZB x NZW)F1 and *FcγRIIB*<sup>-/-</sup> mice were tested for IgG anti-MARCO activity by ELISA (23, 24). In these experiments, sera from 2-, 4-, 6-, and up to 14-mo-old mice were tested, spanning the development of disease that starts at 5 mo of age in (NZB x NZW)F1 mice and at 3 mo of age in *FcγRIIB*<sup>-/-</sup> mice, as determined by end-organ inflammation resulting in pathology such as proteinuria and increased BUN levels. There is also an accumulation of autoantibodies such as IgG anti-DNA levels (Fig. 3, A and C) and anti-C1q (Fig. S2, available at <http://www.jem.org/cgi/content/full/jem.20070600/DC1>). Interestingly, significant levels of IgG anti-MARCO antibodies could be detected as early as 2 mo and peaked at 6–9 mo (Fig. 3, B and D). The binding of the autoimmune sera to MARCO could be blocked by adding an antibody toward the ligand-binding domain (Fig. 3 E)

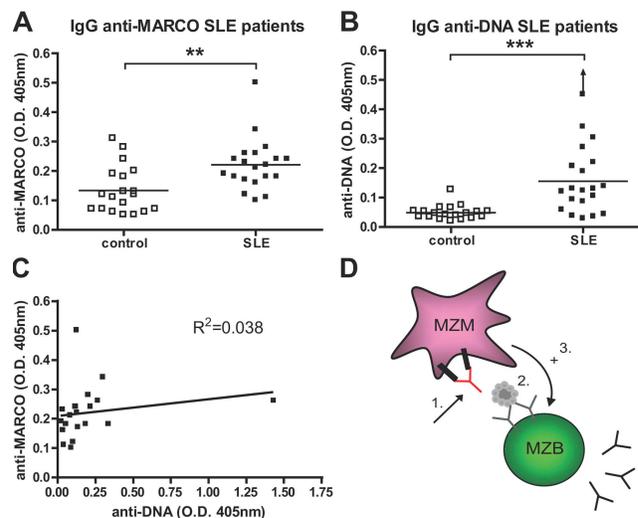


**Figure 4. Anti-MARCO antibodies affect TI-2 B cell responses in C57BL/6 mice.** WT mice were injected with 5  $\mu$ g of the TI-2 antigen TNP-Ficoll alone ( $n = 7$ ) or with 100  $\mu$ g rat IgG anti-MARCO ( $n = 7$ ) or a rat isotypic control ( $n = 4$ ). The data are shown for IgG (A), IgM (B), and IgG3 (C) anti-TNP response pre-immune (PI) at days 5 and 14. In A, data are presented as mean  $\pm$  SEM, and in B and C, individual data and mean are shown. \*,  $P < 0.05$  (nonparametric Mann-Whitney U-test. TI-2 plus anti-MARCO compared with TI-2 or TI-2 plus control group).

and was not affected by treatment with DNase I (Fig. S3). The presence of anti-MARCO autoantibodies in (NZB x NZW)F1 mice was further confirmed by staining transfected cells expressing either the MARCO or SR-A protein (Fig. 3 F), implicating class A scavenger receptors as autoantigens in SLE. The presence of antibodies toward MARCO and SR-A before the onset of disease in (NZB x NZW)F1 and *FcγRIIB*<sup>-/-</sup> mice is compatible with a mechanism where reactivity toward these receptors results in a decreased local clearance of apoptotic cells in the spleen. In addition, i.v. injection of anti-MARCO antibodies has been shown to lead to migration of MZBs, suggesting that these autoantibodies could have dual effects, both activating MZBs and blocking clearance, giving increased access to self-antigens (25). To address whether anti-MARCO antibodies are involved in direct regulation of MZB activation and innate immune responses, we injected them together with the TI-2 antigen TNP-ficoll, which has been shown to be MZB dependent (26). We found that anti-MARCO antibodies, but not the isotype control, enhanced the response (Fig. 4 A), and especially the early MZB-related subclasses IgM and IgG3 (Fig. 4, B and C). This suggests that anti-MARCO reactivity has a direct and T cell-independent effect on the activation threshold and response of MZB cells.

Class A scavenger receptors are highly conserved among species, and MARCO has a 74% amino acid identity between mice and man (7). This led us to investigate whether patients newly diagnosed with SLE also displayed antibodies toward these receptors. When we tested 20 SLE patients and 19 healthy controls, the patients showed significantly ( $P = 0.0035$ ) higher reactivity toward the MARCO protein (Fig. 5 A). The patient group was selected to reflect an early onset of the disease and had low but significant levels of anti-DNA antibodies (Fig. 5 B). Even so, the anti-DNA levels did not correlate with the anti-MARCO levels, suggesting a difference in the regulation that may reflect the situation in the mice (Fig. 5 C). It remains to be determined whether anti-scavenger receptor antibodies also appear before the onset of clinical disease in humans, as the mouse data indicate. The fact that the MZB compartment contains memory B cells both in mouse and man (27) suggests that anti-scavenger receptor antibodies may be involved in the characteristic flares of SLE disease activity. Another possible connection to SLE-related complications is that MARCO and SR-A are receptors for oxidized low-density lipoprotein. This connection could possibly explain why up to 54% of patients with SLE develop cardiovascular disease (28). The association with cardiovascular disease has been suggested to be correlated with impaired clearance of apoptotic cells, but not with any typical measures of SLE activity (28). Our data show that anti-scavenger receptor antibodies are an attractive candidate for such a marker.

In conclusion, herein we describe a novel mechanism for autoimmunity where MZMs regulate the early access and response to apoptotic cells in the spleen through class A scavenger receptors. As a consequence, lack of these receptors in



**Figure 5. SLE patients have circulating anti-MARCO antibodies.**

(A) IgG anti-MARCO reactivity is found in sera from SLE patients. sMARCO or blocking buffer-coated ELISA plates were incubated with sera from SLE patients ( $n = 20$ ) and healthy individuals ( $n = 19$ ). Data are shown as anti-MARCO data minus anti-block buffer data to reduce the level of binding to the block buffer. (B) IgG anti-DNA activity in SLE patients and healthy individuals was measured by ELISA. The patient indicated by an arrow had an  $OD^{405}$  value of 1.44. (C) Correlation between anti-MARCO and anti-DNA levels in the patient group. The two measures do not correlate as deduced by linear regression analysis ( $R^2 = 0.038$ ). (D) A model describing the proposed mechanism where anti-scavenger receptor antibodies influence SLE development by altering the marginal zone response to apoptotic cells. (1.) Anti-scavenger receptor autoantibodies alter the uptake of apoptotic cells by MZMs in the marginal zone leading to an increased local self-antigen load. (2.) Subsequently, auto-reactive B cells have increased access to self-antigens in this area. (3.) In addition, engagement of the MARCO receptor by specific antibodies has the ability to send an activating signal to MZBs, suggesting a dual effect by the anti-scavenger receptor autoantibodies resulting in disease promotion.

mice leads to increased responses to self-antigens. Furthermore, both mice developing spontaneous autoimmune disease and patients with SLE display autoantibodies toward these receptors. In mice, these autoantibodies are found before the onset of disease, and thus anti-scavenger receptor antibodies have potential as predictive markers for disease. We propose that these autoantibodies lower tolerance to nuclear antigens, opening up for subsequent B cell activation by apoptotic cells, giving antibody responses such as anti-DNA that ultimately lead to disease (Fig. 5 D).

## MATERIALS AND METHODS

**Mice.** Mice were age and sex matched and kept and bred under pathogen-free conditions according to local ethical guidelines. *SR-A*<sup>-/-</sup>, *MARCO*<sup>-/-</sup>, and *DKO* mice (15, 16) were backcrossed to the C57BL/6 strain for >10 generations. (NZB x NZW)F1 mice were from The Jackson Laboratory, and *FcγRIIB*<sup>-/-</sup> mice were generated at The Rockefeller University (24). In all studies except noted, WT mice were of the C57BL/6 strain. (NZB x NZW)F1 mice were housed at University of Geneva, and *FcγRIIB*<sup>-/-</sup> mice were housed at The Rockefeller University. All other mice were maintained

at the Medical Biochemistry and Biophysics (MBB) animal facility, and the experiments were approved by the local ethical committee (the North Stockholm district court).

#### Apoptosis induction and injections of apoptotic cells and TNP-Ficoll.

Syngeneic thymocytes were prepared with a cell strainer (Becton Dickinson) in PBS. The cells were cultured for 6 h in RPMI 1640 supplemented with 10% bovine serum, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin (Invitrogen), and 1 µM dexamethasone (Sigma-Aldrich) at a concentration of  $10^7$  cells/ml. The cells were harvested and thoroughly washed three times with sterile PBS. The apoptotic phenotype was evaluated with annexin V-FITC and propidium iodine staining (Becton Dickinson) in a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson). Age- and sex-matched WT (C57BL/6), SR-A<sup>-/-</sup>, MARCO<sup>-/-</sup>, and DKO mice ( $n = 8$  per genotype) were immunized weekly for 4 wk with  $10^7$  apoptotic cells in sterile PBS i.v. in the tail vein (17). Serum samples were collected weekly from the tail artery starting 2 d before the first injection. For TI-2 antigen responses, age- and sex-matched C57BL/6 mice ( $n = 4-7$  per group) were injected i.v. with 5 µg of the TI-2 antigen 2,4,6-trinitrophenol (TNP)-Ficoll (Biosearch) together with 100 µg of the rat IgG1 anti-MARCO monoclonal antibody ED31 (provided by G. Kraal, Vrije Universiteit, Amsterdam, Netherlands) or isotype control (SouthernBiotech). Anti-TNP response was measured with ELISA plates (Nunc) coated with 10 µg/ml TNP-BSA (Biosearch) and alkaline phosphate-conjugated anti-mouse antibodies (SouthernBiotech) using standard methodology. The samples were run in duplicates and corrected for background binding.

**Immunohistochemistry and anti-DNA responses.** Syngeneic thymocytes were prepared and stained with 2 µM PKH26 (Sigma-Aldrich) as described by the manufacturer before induction of apoptosis.  $6 \times 10^7$  cells were injected i.v. into BALB/c mice ( $n = 4$ ). Spleens were collected 45 min and 5 h later and frozen in OCT medium (Sakuru). 6-µm-thin sections were cut in a cryostat microtome. After overnight drying, the slides were fixed in acetone and stored at -75°C. Before staining, slides were blocked with 5% goat serum (DakoCytomation) and 4% BSA in PBS. The following antibodies were used: rat anti-MARCO (29), rat anti-SR-A unlabeled and biotinylated (Serotec), anti-B220-bio and anti-CD11c-FITC (Becton Dickinson), and anti-rat Alexa 488 and streptavidin-Qdot605 (Invitrogen). Images were collected using a confocal laser scanning microscope (TCS SP2; Leica) equipped with one argon and two HeNe lasers. Anti-DNA autoantibodies were measured as described previously (24). In brief, ELISA plates were precoated with methylated BSA and then coated with calf thymus DNA (Sigma-Aldrich). After blocking, serum samples were added. Anti-DNA reactivity was measured with alkaline phosphate-conjugated anti-mouse IgG, IgM, IgG<sub>1</sub>, IgG<sub>2a</sub>, IgG<sub>2b</sub>, and IgG<sub>3</sub> antibodies (SouthernBiotech). All samples were run in duplicates and corrected for background binding. Hep2000 slides (Immuno Concept) were used for ANA assay as described by the manufacturer.

**Binding assays.** Chinese hamster ovary (CHO) cells were transfected with mouse SR-A, MARCO, or a control vector as described previously (30). Apoptotic cells were added in a ratio of 5:1 or 10:1 to transfected cells in DMEM. After a 1-h incubation at 37°C, the cells were washed five times with PBS and processed as described previously (30). The cells were stained for MARCO and SR-A and incubated with Alexa 488-conjugated secondary antibody and DAPI (Invitrogen). Binding was quantified by counting the amount of apoptotic cells binding to transfected or nontransfected cells. Values are the average of two independent experiments for each construct ( $n = 2$ , error bars). Cells were counted by two independent observers. An average of 50 transfected cells were counted on the same slide as the nontransfected cells working as an internal control. The transfection efficacy was 10–15%. Binding was detected with a Leica DMRB microscope coupled to a Retiga Exi Cooled camera.

**Anti-scavenger receptor responses.** Soluble mouse MARCO was purified as described previously (31). MaxiSorp 96-well plates (Nunc) were

coated with 1–2 µg/ml sMARCO in PBS overnight at 4°C. Plates were washed five times with PBS plus 0.05% Tween 20 and blocked with an excess of blocking buffer for 2 h at room temperature (RT). Blocking buffer was tapped off and serum samples were added diluted in blocking buffer followed by a 2-h incubation in RT. The plates were then washed as above, and the following secondary antibodies were added: anti-human IgG-horseradish peroxidase (DAKO) or anti-mouse IgG-AP (SouthernBiotech). After a 1-h incubation at RT, plates were washed and substrate was added. All samples were run in duplicates and corrected for background binding.

**Patients.** 20 SLE patients classified by the American College of Rheumatology criteria (32) and 19 matched healthy individuals were selected. Sera from young SLE patients (median age 35) with low anti-DNA titres were chosen because mouse data indicate that anti-MARCO reactivity can be found early in development of the disease. The use of patient samples was approved by the local ethical committee (the North Stockholm district court).

**Statistical analysis.** Nonparametric Mann-Whitney U test was performed using Statistica software (StatSoft Inc.) and GraphPad Prism 4 (GraphPad Software, Inc.).  $P < 0.05$  was considered significant.

**Online supplemental material.** Fig. S1 shows basal level of apoptotic cells and clearance of apoptotic cells from the blood in scavenger receptor DKO mice and controls. Fig. S2 shows a time course of spontaneous anti-C1q levels in (NZBxNZW)F1 and FcγRIIB<sup>-/-</sup> mice. Fig. S3 demonstrates that binding of (NZBxNZW)F1 sera to MARCO is not due to DNA contamination. The online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20070600/DC1>.

We thank Drs. R. Guinamard, F. Nimmerjahn, and T. McGaha for reagents, scientific discussions, and suggestions; Dr. G. Kraal for the anti-mouse MARCO monoclonal antibody ED31 hybridoma; Dr. L. Rönnblom for important input; the staff at the MBB animal facility for taking good care of the mice; Dr. M. Sankala and J. Pagan for reagents; Dr. P. Magnusson and Dr. H. Grönlund for discussions and technical as well as statistical expertise; and S. Lind and E. Lundgren for critical review of the manuscript.

This work was supported by the Swedish Research Council, the National Institutes of Health, the Lundberg Foundation, the Ronald McDonald Foundation, Grönwall's Foundation, the Magnus Bergvall Foundation, the Sven Jerring Foundation, the Swedish Diabetes Association, the Swedish Medical Society, Agnes and Mac Rudberg's Foundation, the Queen Silvia Jubilee Foundation, the Swedish Juvenile Diabetes Foundation, the Swedish Cancer and Allergy Foundation, and the Swiss National Foundation for Scientific Research.

The authors have no conflicting financial interests.

Submitted: 26 March 2007

Accepted: 16 August 2007

## REFERENCES

- Lopes-Carvalho, T., and J.F. Kearney. 2004. Development and selection of marginal zone B cells. *Immunol. Rev.* 197:192–205.
- Kraal, G., and R. Mebius. 2006. New insights into the cell biology of the marginal zone of the spleen. *Int. Rev. Cytol.* 250:175–215.
- Martin, F., and J.F. Kearney. 2002. Marginal-zone B cells. *Nat. Rev. Immunol.* 2:323–335.
- Li, Y., H. Li, D. Ni, and M. Weigert. 2002. Anti-DNA B cells in MRL/lpr mice show altered differentiation and editing pattern. *J. Exp. Med.* 196:1543–1552.
- Enzler, T., G. Bonizzi, G.J. Silverman, D.C. Otero, G.F. Widhopf, A. Anzelon-Mills, R.C. Rickert, and M. Karin. 2006. Alternative and classical NF-κappa B signaling retain autoreactive B cells in the splenic marginal zone and result in lupus-like disease. *Immunity.* 25:403–415.
- Mandik-Nayak, L., J. Racz, B.P. Sleckman, and P.M. Allen. 2006. Autoreactive marginal zone B cells are spontaneously activated but lymph node B cells require T cell help. *J. Exp. Med.* 203:1985–1998.

7. Elomaa, O., M. Kangas, C. Sahlberg, J. Tuukkanen, R. Sormunen, A. Liakka, I. Thesleff, G. Kraal, and K. Tryggvason. 1995. Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell*. 80:603–609.
8. Kim, S.J., D. Gershov, X. Ma, N. Brot, and K.B. Elkon. 2003. Opsonization of apoptotic cells and its effect on macrophage and T cell immune responses. *Ann. NY Acad. Sci.* 987:68–78.
9. Casciola-Rosen, L.A., G. Anhalt, and A. Rosen. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* 179:1317–1330.
10. Morelli, A.E., A.T. Larregina, W.J. Shufesky, A.F. Zahorchak, A.J. Logar, G.D. Papworth, Z. Wang, S.C. Watkins, L.D. Falo Jr., and A.W. Thomson. 2003. Internalization of circulating apoptotic cells by splenic marginal zone dendritic cells: dependence on complement receptors and effect on cytokine production. *Blood*. 101:611–620.
11. Steinman, R.M., S. Turley, I. Mellman, and K. Inaba. 2000. The induction of tolerance by dendritic cells that have captured apoptotic cells. *J. Exp. Med.* 191:411–416.
12. Peiser, L., and S. Gordon. 2001. The function of scavenger receptors expressed by macrophages and their role in the regulation of inflammation. *Microbes Infect.* 3:149–159.
13. Platt, N., H. Suzuki, Y. Kurihara, T. Kodama, and S. Gordon. 1996. Role for the class A macrophage scavenger receptor in the phagocytosis of apoptotic thymocytes in vitro. *Proc. Natl. Acad. Sci. USA*. 93:12456–12460.
14. Hanayama, R., M. Tanaka, K. Miyasaka, K. Aozasa, M. Koike, Y. Uchiyama, and S. Nagata. 2004. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science*. 304:1147–1150.
15. Suzuki, H., Y. Kurihara, M. Takeya, N. Kamada, M. Kataoka, K. Jishage, O. Ueda, H. Sakaguchi, T. Higashi, T. Suzuki, et al. 1997. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature*. 386:292–296.
16. Chen, Y., T. Pikkarainen, O. Elomaa, R. Soininen, T. Kodama, G. Kraal, and K. Tryggvason. 2005. Defective microarchitecture of the spleen marginal zone and impaired response to a thymus-independent type 2 antigen in mice lacking scavenger receptors MARCO and SR-A. *J. Immunol.* 175:8173–8180.
17. Mevorach, D., J.L. Zhou, X. Song, and K.B. Elkon. 1998. Systemic exposure to irradiated apoptotic cells induces autoantibody production. *J. Exp. Med.* 188:387–392.
18. Nimmerjahn, F., and J.V. Ravetch. 2005. Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science*. 310:1510–1512.
19. Kono, D.H., and A.N. Theofilopoulos. 2006. Genetics of SLE in mice. *Springer Semin. Immunopathol.* 28:83–96.
20. Hogarth, M.B., J.H. Slingsby, P.J. Allen, E.M. Thompson, P. Chandler, K.A. Davies, E. Simpson, B.J. Morley, and M.J. Walport. 1998. Multiple lupus susceptibility loci map to chromosome 1 in BXSB mice. *J. Immunol.* 161:2753–2761.
21. Acton, S., D. Resnick, M. Freeman, Y. Ekkel, J. Ashkenas, and M. Krieger. 1993. The collagenous domains of macrophage scavenger receptors and complement component C1q mediate their similar, but not identical, binding specificities for polyanionic ligands. *J. Biol. Chem.* 268:3530–3537.
22. Trouw, L.A., M.A. Seelen, R. Visseren, J.M. Duijs, H. Benediktsson, E. de Heer, A. Roos, C. van Kooten, and M.R. Daha. 2004. Anti-C1q autoantibodies in murine lupus nephritis. *Clin. Exp. Immunol.* 135:41–48.
23. Burnet, F.M., and M.C. Holmes. 1965. The natural history of the NZB/NZW F1 hybrid mouse: a laboratory model of systemic lupus erythematosus. *Australas. Ann. Med.* 14:185–191.
24. Bolland, S., and J.V. Ravetch. 2000. Spontaneous autoimmune disease in Fc(gamma)RIIB-deficient mice results from strain-specific epistasis. *Immunity*. 13:277–285.
25. Karlsson, M.C., R. Guinamard, S. Bolland, M. Sankala, R.M. Steinman, and J.V. Ravetch. 2003. Macrophages control the retention and trafficking of B lymphocytes in the splenic marginal zone. *J. Exp. Med.* 198:333–340.
26. Guinamard, R., M. Okigaki, J. Schlessinger, and J.V. Ravetch. 2000. Absence of marginal zone B cells in Pyk-2-deficient mice defines their role in the humoral response. *Nat. Immunol.* 1:31–36.
27. Weller, S., M.C. Braun, B.K. Tan, A. Rosenwald, C. Cordier, M.E. Conley, A. Plebani, D.S. Kumararatne, D. Bonnet, O. Tournilhac, et al. 2004. Human blood IgM “memory” B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. *Blood*. 104:3647–3654.
28. Maksimowicz-McKinnon, K., L.S. Magder, and M. Petri. 2006. Predictors of carotid atherosclerosis in systemic lupus erythematosus. *J. Rheumatol.* 33:2458–2463.
29. van der Laan, L.J., M. Kangas, E.A. Dopp, E. Broug-Holub, O. Elomaa, K. Tryggvason, and G. Kraal. 1997. Macrophage scavenger receptor MARCO: in vitro and in vivo regulation and involvement in the antibacterial host defense. *Immunol. Lett.* 57:203–208.
30. Brannstrom, A., M. Sankala, K. Tryggvason, and T. Pikkarainen. 2002. Arginine residues in domain V have a central role for bacteria-binding activity of macrophage scavenger receptor MARCO. *Biochem. Biophys. Res. Commun.* 290:1462–1469.
31. Sankala, M., A. Brannstrom, T. Schulthess, U. Bergmann, E. Morgunova, J. Engel, K. Tryggvason, and T. Pikkarainen. 2002. Characterization of recombinant soluble macrophage scavenger receptor MARCO. *J. Biol. Chem.* 277:33378–33385.
32. Tan, E.M., A.S. Cohen, J.F. Fries, A.T. Masi, D.J. McShane, N.F. Rothfield, J.G. Schaller, N. Talal, and R.J. Winchester. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum.* 25:1271–1277.